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DNA Methylation at Promoter Regions Regulates the Timing of Gene Activation in *Xenopus laevis* Embryos

Irina Stancheva,* Osman El-Maarri,† Joern Walter,‡ Alain Niveleau,§ and Richard R. Meehan*,[1]

**Department of Biomedical Sciences, Genes and Development Group, The University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, United Kingdom; †Institute of Experimental Haematology and Transfusion Medicine, Sigmund-Freud Strasse 25, 53105 Bonn, Germany; ‡Department of Genetics, University of Saarland, D-66041 Saarbrücken, Germany; and §Molecular and Structural Virology Unit, Universite J. Fourier de Grenoble, 38706 La Tronche, France*

The levels of genomic DNA methylation in vertebrate species display a wide range of developmental dynamics. Here, we show that in contrast to mice, the paternal genome of the amphibian, *Xenopus laevis,* is not subjected to active demethylation of 5-methyl cytosine immediately after fertilization. High levels of methylation in the DNA of both oocyte and sperm are maintained in the early embryo but progressively decline during the cleavage stages. As a result, the *Xenopus* genome has its lowest methylation content at the midblastula transition (MBT) and during subsequent gastrulation. Between blastula and gastrula stages, we detect a loss of methylation at individual *Xenopus* gene promoters (*TFIIIA*, *Xbra,* and *c-Myc II*) that are activated at MBT. No changes are observed in the methylation patterns of repeated sequences, genes that are inactive at MBT, or in the coding regions of individual genes. In embryos that are depleted of the maintenance methyltransferase enzyme (xDnmt1), these developmentally programmed changes in promoter methylation are disrupted, which may account for the altered patterns of gene expression that occur in these embryos. Our results suggest that DNA methylation has a role in regulating the timing of gene activation at MBT in *Xenopus laevis* embryos.

Key Words: **Xenopus; development; demethylation; xDnmt1.**

INTRODUCTION

In mammals, DNA methylation is essential for the regulation of a variety of biological processes, including genomic imprinting, X-chromosome inactivation, gene regulation, and protection against invading DNA molecules (Colot and Rossignol, 1999; Jaenisch, 1997). In mice, remodeling of the somatic methylation patterns initially occurs during gametogenesis (Sanford *et al.*, 1987). Further changes are observed soon after fertilization, when the paternal pronucleus becomes specifically demethylated by an activity present in the oocyte cytoplasm (Mayer *et al.*, 2000; Oswald *et al.*, 2000). During the cleavage stages of preimplantation mouse embryos, additional genome-wide loss of methylation occurs. This is attributed to cytoplasmic sequestration of the maintenance methyltransferase, Dnmt1 (Monk *et al.*, 1987; Kafri *et al.*, 1992; Mertineit *et al.*, 1998). Although the biological function of demethylation events is not completely understood, it has been proposed that demethylation of the paternal genome by the egg cytoplasm is related to the establishment of imprinted marks at specific gene loci (Oswald *et al.*, 2000). It is thought that imprinting in placental animals is an evolutionary adaptation that maintains a fine balance between the competing requirements of the maternal and paternal genomes in the regulation of embryo size and subsequent survival (Moore and Reik, 1996; Tilghman, 1999). Other vertebrate species, such as amphibia, fish, birds, and marsupials, which do not depend on the resources of the maternal organism for embryo development, lack imprinted genes (Corley-Smith *et al.*, 1999; Yamada *et al.*,

[1] To whom correspondence should be addressed. Fax: 0131-650-3714. E-mail: Richard.Meehan@ed.ac.uk.

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1999). Despite these significant differences between mammals and other species, the components of the methylation machinery are highly conserved in all vertebrates (Bird and Wolffe, 1999). Loss of DNA methylation, by inactivation of the maintenance methyltransferase (Dnmt1), is associated with severe developmental defects in *Xenopus* and zebrafish or early embryonic lethality in mice (Li *et al.*, 1996; Martin *et al.*, 1999; Stancheva and Meehan, 2000). No detectable changes in methylation levels were observed at repeated DNA sequences during development of zebrafish from blastula stages (2.2 h) onward (Macleod *et al.*, 1999; Martin *et al.*, 1999), suggesting that global demethylation is not an obligate requirement for early vertebrate embryogenesis.

The *Xenopus* genome, similar to other vertebrates, is methylated at the fifth position of cytosine at CpG dinucleotides. DNA methylation contributes to the transcriptional silencing during the first 12 cleavages of the zygote, and loss of this epigenetic modification is associated with premature activation of developmentally decisive genes and apoptosis of embryo cells (Stancheva and Meehan, 2000; Stancheva *et al.*, 2001). The *Xenopus* genome is pseudotetraploid and, compared to mammals, is CG-poor (Cooper *et al.*, 1983; Gardiner-Garden and Frommer, 1987). The majority of sequenced gene promoters lack the CpG island structure that is typical for mice and humans. Where present, *Xenopus* CpG islands are usually 50% G + C-rich, short in length (between 200 and 800 bp), and most often flank transcription initiation sites. We wished to know whether global changes in DNA methylation levels or specific remodeling of methylation patterns takes place postfertilization and during the early development of *Xenopus laevis*. Our data demonstrate that the *Xenopus* genome does not undergo global demethylation at any stage of development. However, we observed specific changes in the methylation pattern of individual gene promoters which are activated at MBT in toad embryos. These developmentally programmed changes are disrupted in embryos depleted from the maintenance methyltransferase enzyme, Dnmt1, and lead to inappropriate expression patterns of genes that are involved in the formation of embryonic germ layers (Stancheva and Meehan, 2000). Our results support a model where, by the repressive effect of DNA, methylation at gene promoters is utilized to regulate the precise timing of gene expression at MBT.

MATERIALS AND METHODS

Xenopus CpG Island Analysis

To map CpG islands on *Xenopus* promoters, we used Web Gene tools at http://liter25.itba.mi.cnr.it/genebin/wwwcpg.pl. The Obs./Exp. ratio for CpGs at the analyzed promoters was 0.66 for *Xbra* promoter (GpG island −177 to + 299); 0.61 for *c-Myc II* promoter (CpG island −458 to + 1186); 0.71 for *TFIIIA* promoter (CpG island −82 to + 121), and for *cardiac Actin* promoter Obs./Exp. = 0.4 (no CpG island).

5mC Antibody Immunostaining

Sperm and oocyte nuclei were prepared as described elsewhere (Lemaitre *et al.*, 1995; Sive *et al.*, 2000). *Xenopus* embryos were obtained from natural mating of wild-type or albino frogs and staged according to Nieuwkoop and Faber (1994). Nuclei from one-, two-, and four-cell embryos were attached to glass cover slips by centrifugation (1500 rpm) through a 30% glycerol/PBS cushion. Later-stage embryos were first dissociated to single cells by incubation in CMF medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 7.5 mM Tris, pH 7.6) prior to centrifugation through a glycerol cushion. The nuclei were briefly fixed with 0.25% formaldehyde in PBS, and the DNA was denatured at 80°C in 70% formamide, 2× SSC. After two brief washes with PBS, the nuclei were incubated with 1:10 dilution of 5mC antibody for 1 h, sequentially with the secondary anti-mouse IgG FITC-conjugated antibody (1:500) for 15 min, washed, and mounted in 90% glycerol/PBS to microscope slides. Images were collected by using a Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany).

Extract Preparation

Xenopus egg extracts was prepared according to published procedures (Murray, 1991). Sperm or oocyte nuclei were incubated in the extracts from 15 to 120 min prior to immunohistochemical analysis or DNA preparation.

Bisulfite Sequencing

Bisulfite sequencing was performed as described (Olek *et al.*, 1996). The primers used for PCR amplification of bisulfite-modified sequences were: for *TFIIIA* promoter f, GGTGGGAATTAGGGAATGGT; r, CCCCCGCCTATCTTTACAAC; for *Xbra* promoter f, GATATAGGTGTAAATATTTGGGG; r, CATAATCATAATAATATAAATCTACAC; for *c-Myc II* promoter f, GGTTGTTAGGGGAATTTGG; r, TCCTTTTAACTCTTCCACTAAC; for *car.Actin* promoter f, TTTAATAGAGTGTAAGTTTTGAG; r, CCAATAAAATCTTAATTTATCCC; for *Satellite1* f, CAAGCA TTTGAGAAGCTGACTAG; r, GGCAAGGTGCTAGATGGGAAAGCC; for *Xbra* gene f, CTTATTCCTAATGGT GGGTCAT; r, CATATTACTCACAAACCATAA; for *c-Myc II* gene (exon one +566 to +1145) f, GGGAGAGTTATAGGTTATGG; r, CCCTCCCAAAAATTCAATCA; for *car.Actin* gene (end of exon one, intron one +1222 to +1725) f, GGGTAGGTAGATTGGGTTGGT; r, CTTATACAACTATATCCTCCTATC. The PCR fragments, cloned into pGEM-T easy vector (Promega), had sizes between 200 and 600 bp (see Figs. 3–5) and contained originally: 12 CpGs for *TFIIIA* promoter, 16 CpGs for *Xbra* promoter; 11 CpGs for *c-MycII* promoter; 12 CpGs for *car.Actin* promoter, 16 CpGs for *Satellite1*, 7 CpGs for *Xbra* gene, 15 CpGs for *c-MycII* gene, and 12 CpGs for *car.Actin* gene. The number of sequenced individual clones derived from sperm, oocyte, blastula (stage 6.5), and gastrula (stage 12.5) developmental stages was: 15 for *Xbra* promoter, 10 for *c-Myc*, *carActin* promoters, and *Satellite1* sequences, and 5 for *Xbra*, *c-Myc*, and *car.Actin* coding gene regions.

Southern Blot and Hybridization

DNA was isolated from wild-type and *Dnmt1*-depleted staged embryos according to standard procedure (Sive *et al.*, 2000). The only modification of the standard protocol was the extraction of embryo lysates with 1,1,2-trichlorotrifluoroethane prior to protein-

ase K digestion, which helped to remove the majority of yolk and pigment proteins. All samples were divided in two and digested with either *Hpa*II or *Msp*I restriction enzymes. Digested DNA was electrophoresed in 1% native agarose gels and transferred to Byodine B membrane (Pall Ltd). Labeling and hybridization with a 1.2-kb *Pst*I fragment of the *Xbra* promoter, a 770-bp *Bam*HI fragment of *cardiac Actin* promoter, and a 1.2-kb of mitochondrial ND1 ORF were performed according to manufacturer's protocols (Amersham and Pall).

RESULTS

Demethylation of Parental Genomes Does Not Occur in Early Xenopus Zygote

Both *Xenopus* sperm and oocyte nuclei are highly methylated, and these relatively high levels are maintained in the early embryo (Bird *et al.*, 1981;Harland, 1982; Stancheva and Meehan, 2000). To test whether the initial methylation patterns present in the gametes undergo any changes after fertilization, we performed immunostaining of nuclei derived from *Xenopus* oocytes, one- and two-cell embryos, and early blastula and gastrula stages (Fig. 1) with a 5mC antibody. The antibody stained equally well oocyte nuclei and those from 1-, 2-, 4-, and 16-cell embryo (Figs. 1A–1D), but it failed to detect 5mC in stage 7.5–8 blastula nuclei from a control embryo depleted of the maintenance DNA methyltransferase, *xDnmt1*, by anti-sense RNA injection (Fig. 1F). The overall intensity and pattern of staining appeared to be very similar during the first cleavages of the *Xenopus* zygote (Figs. 1A–1C). Unlike DAPI staining (Figs. 1G–1L), the 5mC antibody fluorescence is not uniform and may be indicative of distinct nuclear domains or clustering of methylated sequences. During the early rapid cleavage stages, *Xenopus* chromosomes have the ability to replicate and segregate as independent units called "karyomeres" without complete reconstitution of the interphase cellular nucleus (Lemaitre *et al.*, 1998). The karyomeres from a 16-cell embryo were also stained by the 5mC antibody (Fig. 1D), but in this case, the pattern was similar to that observed with DAPI staining (compare Figs. 1D and 1J). Nuclei derived from stage 12.5 gastrula, however, had less intense and a more diffuse 5mC staining with fewer methylation clusters compared to the earlier stages (Fig. 1E), suggesting that there might be a decrease and remodeling of DNA methylation patterns after MBT and during embryo gastrulation.

In *Xenopus*, the paternal and maternal pronuclei are difficult to distinguish in the one- or two-cell zygote due to the lack of suitable parent of origin markers. Therefore, we performed additional *in vitro* experiments to test whether either of the two genomes, maternal or paternal, is subjected to changes in the 5mC levels induced by exposure to egg cytoplasm. *Xenopus* sperm and oocyte nuclei were prepared and incubated in either meiotic metaphase arrested (CSF) or interphase cytoplasmic egg extracts for 15, 30, 45, and 90 min. After incubation, the nuclei were either stained with 5mC antibody/DAPI or the DNA was purified and analyzed by immuno dot-blotting and by bisulfite sequencing. This last approach allows chromosomal methylation patterns of specific sequences to be directly determined (Olek *et al.*, 1996). As shown in Figs. 2A–2C, sperm nuclei undergo swelling and chromatin decondensation in the interphase egg extract (Figs. 2A–2C), but there is no visible change of the 5mC staining after up to 90 min of incubation, which corresponds to the time of the first zygotic division *in vivo* (Newport and Kirschner, 1982). Exposure to a metaphase-arrested extract also did not lead to any decrease in the intensity of 5mC staining (data not shown). Similar results were obtained with oocyte nuclei incubated in both egg extracts (data not shown, but see Figs. 2G, 3B, and 5B). These experiments, *in vivo* and *in vitro*, were confirmed by genomic DNA dot-blot immunodetection of 5mC (Fig. 2G). The antibody reacted equally well with DNA derived from sperm and oocyte nuclei incubated in egg extracts, and the intensity of the signal did not change with time (Fig. 2G, *in vitro*). In contrast, and as noted previously, the level of 5mC in DNA decreases in early- to midgastrula-stage embryos (Fig. 2G, *in vivo*). From these experiments, we conclude that neither the maternal nor the paternal genomes of the *Xenopus* zygote undergoes any dramatic changes of DNA methylation during the first few hours after fertilization and that the demethylation activity is absent from *Xenopus* egg extracts. On the other hand, there is an approximate 40% decrease in 5mC content in the DNA isolated from embryos collected between early blastula and midgastrula stage (Fig. 2G). This is in agreement with our previous observation that the amount of 5mC as well as *xDnmt1* also progressively decreases during the cleavages of *Xenopus* embryo, reaching its lowest levels around MBT (Stancheva and Meehan, 2000).

Developmentally Regulated Gene Promoters Undergo Remodeling of Methylation Patterns in Gastrula-Stage Embryos

We tested whether individual genes in the paternal or maternal genomes underwent demethylation or remodeling of their methylation patterns in the early embryo or in nuclei incubated in egg extracts. Bisulfite sequencing of DNA, which positively identifies 5mC, was used to analyze the methylated CpGs in the *Xbra*, *c-Myc II*, *TFIIIA*, and *cardiac Actin* gene promoters, in gene coding regions (*Xbra*, *c-Myc II*, and *cardiac Actin*), as well as in the highly repeated *Satellite 1* sequences (Artinger *et al.*, 1997; Lam and Carroll, 1983; Mohun *et al.*, 1986; Principaud and Spohr, 1991). The promoters of *Xbra*, *TFIIIA*, and *c-Myc II* have short (400- to 800-bp) stretches of CpG-rich DNA (CpG island-like) that flank the transcription initiation site (Figs. 3A, 4A, and 5A). The expression of *Xbra* T-box transcription factor and the housekeeping genes *TFIIIA* and *c-Myc* is activated at the MBT during embryo development (Artinger *et al.*, 1997; Principaud and Spohr, 1991; Taylor *et al.*, 1986). *Cardiac Actin* is a tissue-specific gene (Mohun *et al.*, 1986) that begins to be expressed at early neurula stages

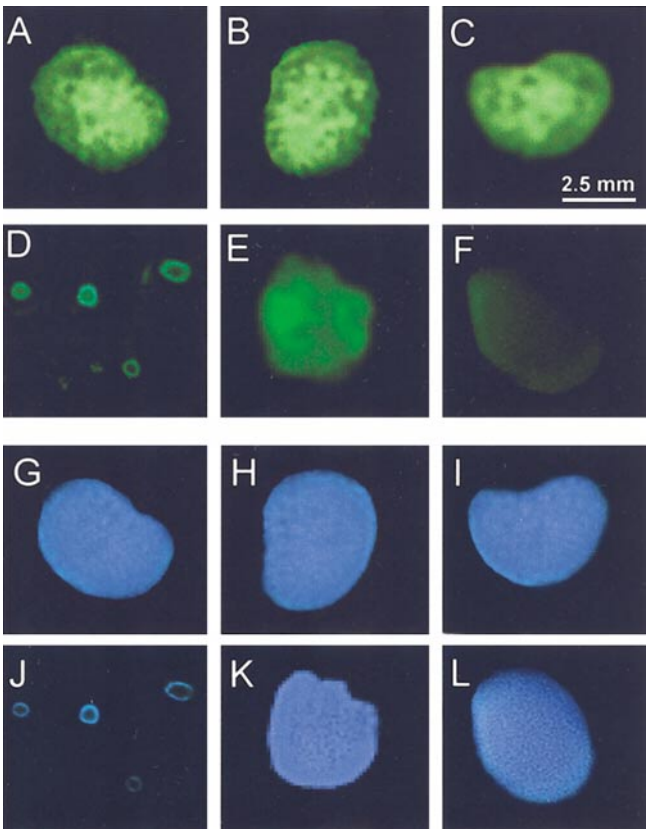

FIG. 1. The *Xenopus* embryo genome does not undergo demethylation after fertilization and during the very early cleavage stages. (A) Oocyte nucleus stained with an antibody against 5mC (green). (B) Nucleus from a one-cell-stage embryo stained for 5mC. (C) Nucleus from a two-cell-stage embryo stained for 5mC. (D) During the cleavage stages (stages 2–8), the chromosomes of the embryo replicate and segregate as independent units (karyomeres) without forming a typical interphase nucleus. Karyomeres from 16-cell embryos also stained well with the antibody against 5mC. (E) Nucleus from a stage 12.5 gastrula embryo stained for 5mC. Note different organization of chromatin clusters compared to (A–C). (F) Nucleus, stained with the antibody against 5mC, from stage 7.5–8 embryo depleted of *xDnmt1* by injection of anti-sense RNA at the two-cell stage. (G–L) Dapi staining of the same nuclei as in (A–F).

(much later compared to *Xbra*, *TFIIIA*, and *c-Myc*). It does not have a CpG island, but there are several CpGs clustered around its transcription start site, which were suitable for bisulfite analysis (Fig. 4C).

Bisulfite sequencing showed that the *Xbra*, *TFIIIA*, and c-*Myc* promoters were highly methylated in oocyte, sperm, and early cleavage-stage embryos, but had lost most of the methylated CpGs in gastrula-stage 12.5 embryos (Figs. 3B, 4B, and 5B). Demethylation was especially prominent in the *Xbra* and *TFIIIA* promoters, where around 70–85% of the methylated CpGs were lost at gastrula stages (Figs. 3 and 5B). Approximately 48% of mCpGs in the *c-Myc* promoter were hypomethylated in gastrula (Fig. 5B). In contrast, the *cardiac Actin* promoter was initially hypomethylated in sperm, oocyte, and blastula-stage embryos, but had more (~30%) methylated CpGs in stage 12.5 gastrulae (Figs. 4D and 5B). In comparison to the promoter sequences, we did not detect any change in the levels of DNA methylation in the coding regions of *Xbra*, *c-Myc*, and *cardiac Actin* genes at the onset of gastrulation by using either Southern blot hybridization (not shown) or bisulfite DNA sequencing (Figs. 5C and 5D). In all cases, we found the body of the *Xbra* gene to be undermethylated at all stages of development, while *c-Myc* and *cardiac Actin* coding gene regions were highly methylated at the analyzed sequences. In addition, we also investigated the 720-bp *Satellite 1* repeat sequences for the presence of 5mC. *Satellite1* has approximately $2–4 \times 10^4$ copies in the *Xenopus* genome and localizes to pericentromeric DNA in half (18 out of 36) of the *Xenopus* chromosomes (Jamrich *et al.*, 1983). We found *Satellite1* to be highly methylated at all stages of development (Fig. 5D), which is similar to the methylation state of other repeated sequences such as the pseudoretrotransposon *Trx 23* (I.S. and R.R.M., unpublished observations).

In summary, bisulfite genomic sequencing experiments were in agreement with the dot-blot assay for 5mC (Fig. 2G), which showed that the overall genomic methylation levels in *Xenopus* embryo during the first six to seven cleavages of the zygote remain relatively stable and high. We were able to specify that the ~40% decrease in the DNA methylation levels that occurs at the onset of gastrulation is due to developmentally programmed remodeling of embryonic methylation patterns at early activated (at MBT) gene promoters but not at gene coding regions or repeated genomic sequences. This suggests that demethylation is targeted to promoters that become active at the MBT.

The Somatic Methylation Patterns Are Disrupted in xDnmt1-Depleted Embryos

The significance of preexisting methylation patterns in the early (pre-MBT) *Xenopus* embryo was highlighted when we analyzed the methylation of the *Xbra* and *cardiac Actin* promoters by *Hpa*II digestion and Southern blot hybridization in embryos that were transiently depleted of the maternal form of the maintenance methyltransferase enzyme (*xDnmt1*) by anti-sense RNA injection (Stancheva and Meehan, 2000). In *Xenopus*, *xDnmt1* is present first as an abundant maternal transcript and protein up to MBT, when the gene starts to be expressed zygotically in developing embryos. Anti-sense *xDnmt1* RNA injection leads to loss of the maternal transcript and protein and complete demethylation of the embryo genome at around stage 6, although it does not affect the zygotic form of the enzyme. Many genes, including *Xbra*, are prematurely expressed in these transiently hypomethylated embryos (Stancheva and Meehan, 2000; D. Dunican, I.S., and R.R.M., unpublished observations).

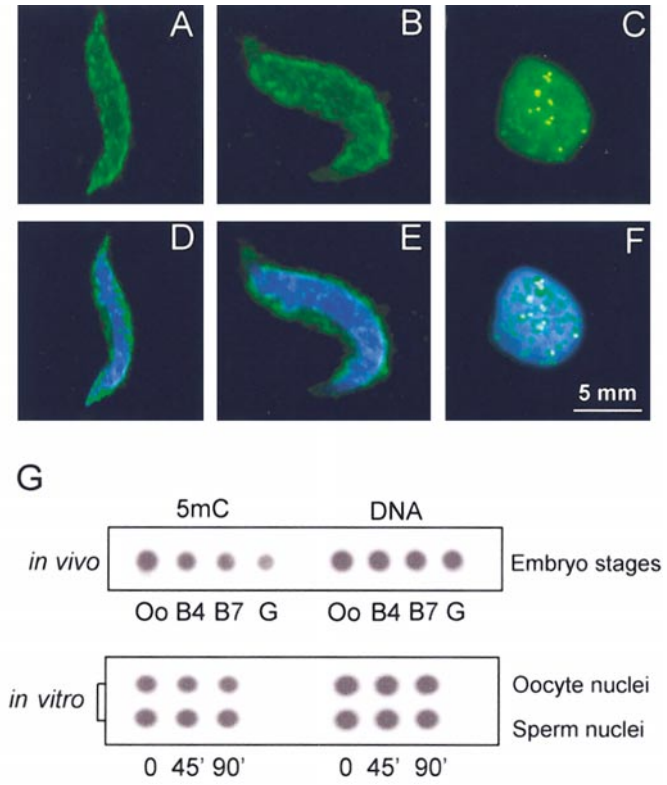

FIG. 2. Demethylation activity is absent from *Xenopus* egg cytoplasm. (A) Sperm nucleus stained with the antibody against 5mC. (B) Sperm nucleus incubated in an interphase egg extract for 45 min still stains well with the 5mC antibody. (C) Sperm nucleus after 90 min of incubation in the extract (corresponding to the time of the first cleavage of the zygote) has the same intensity of 5mC staining as the nuclei in (A) and (B). Note the progressive swelling of the sperm nuclei that reflects chromatin decondensation induced by the egg extract. (D–F) Double fluorescence of the same sperm nuclei as in (A–C), in blue for Dapi and in green for 5mC antibody. (G) Dot blot immunodetection of 5 methyl-cytosine in DNA from oocyte (Oo), blastulae stage 4 (B4), 7 (B7), and stage 12 gastrula (G) *Xenopus* embryos (upper panel left, *in vivo*). The amount of 5mC (5 methyl-cytosine) detected by the antibody progressively decreases toward gastrulation (compared to the hybridization signal for total DNA in the right side of the panel). There is no detectable change in the 5mC content of the oocyte and sperm nuclei incubated in interphase egg extract for 0, 45, and 90 min (lower left panel, *in vitro*). On the right side of the panel, the total DNA in the same spots (as on the left) was detected by DNA hybridization with the *Satellite1* probe.

In agreement with our previous data, the *Xbra* promoter lost methylated CpGs during the cleavage stages in *xDnmt1*-depleted embryos (Fig. 6A, compare *Hpa*II digests of WT to xDnmt1(−) blastula). *Cardiac actin*, as expected, was not methylated in either wild-type or *xDnmt1*-depleted embryos [Fig. 6B, see the *Hpa*II digests of WT and xDnmt(−) blastula]. During gastrulation in wild-type embryos, the *Xbra* promoter remained unmethylated but became *de novo* methylated in *xDnmt1*-depleted embryos [Fig. 4A, compare WT and xDnmt1(−) gastrulae *Hpa*II]. In contrast, although normally methylated in the wild-type gastrula embryos, the *cardiac Actin* promoter failed to be *de novo* methylated after transient depletion of *xDnmt1* [Fig. 6B, compare WT and xDnmt1(−) gastrula *Hpa*II]. This suggests that, in *xDnmt1*-depleted embryos, loss of methylation during early stages of development is able to disrupt the developmentally programmed changes in methylation of the *Xbra* and *cardiac Actin* gene promoters. Other sequences, such as mitochondrial DNA (Fig. 6C) and the CpG island region in the promoter of the fibronectin gene (data not shown), remain unmethylated during development and were not affected by loss of *xDnmt1*.

DISCUSSION

5mC antibody staining and bisulfite sequencing of DNA from oocyte, sperm, and early cleavage-stage embryo nuclei demonstrated that both *Xenopus* parental genomes remain methylated before and after the first cleavage of the zygote, implying that there is no demethylation activity present in *Xenopus* egg cytoplasm. This observation is in agreement with a proposed idea that imprinting mechanisms and active demethylation of the paternal genome arose as the result of a genetic conflict between parents over the allocation of maternal resources to mammalian embryos (Reik and Walter, 2001). One prediction derived of this model is that, in oviparous animals, where there is no asymmetry in the contribution to embryo growth by each parent, the genetic conflict will be absent. This is exactly what was observed in *Xenopus*, zebrafish, and chickens (Corley-Smith *et al.*, 1999; Macleod *et al.*, 1999; O'Neill *et al.*, 2000; Yamada *et al.*, 1999). In addition, we did not find a global wave of demethylation in *Xenopus* embryos, although the genomic levels of 5mC can decrease by up to 40% at the onset of gastrulation (Radomski *et al.*, 1999; Stancheva and Meehan, 2000). This is accompanied by a remodeling of methylation patterns at individual gene promoters, while gene coding regions and repeated sequences remain heavily methylated, with the exception of the *Xbra* coding region, at all stages of development.

In *Xenopus* embryos, zygotic transcription begins after approximately 12 cell divisions of the fertilized egg. DNA methylation contributes significantly to the maintenance of transcriptional silencing during the cleavage stages and, together with the lack of imprinting, may account for the absence of demethylation and remodeling of methylation patterns in the early *Xenopus* embryos. Toward the MBT, blastula-stage embryos gradually, but not uniformly, lose methylation at specific gene promoters. In addition to RNA polymerase II genes, we found that the RNA polymerase I-regulated ribosomal gene (rDNA) cluster is prematurely activated in *Dnmt1*-depleted embryos (Stancheva and Meehan, 2000). Normally, its promoter region is heavily

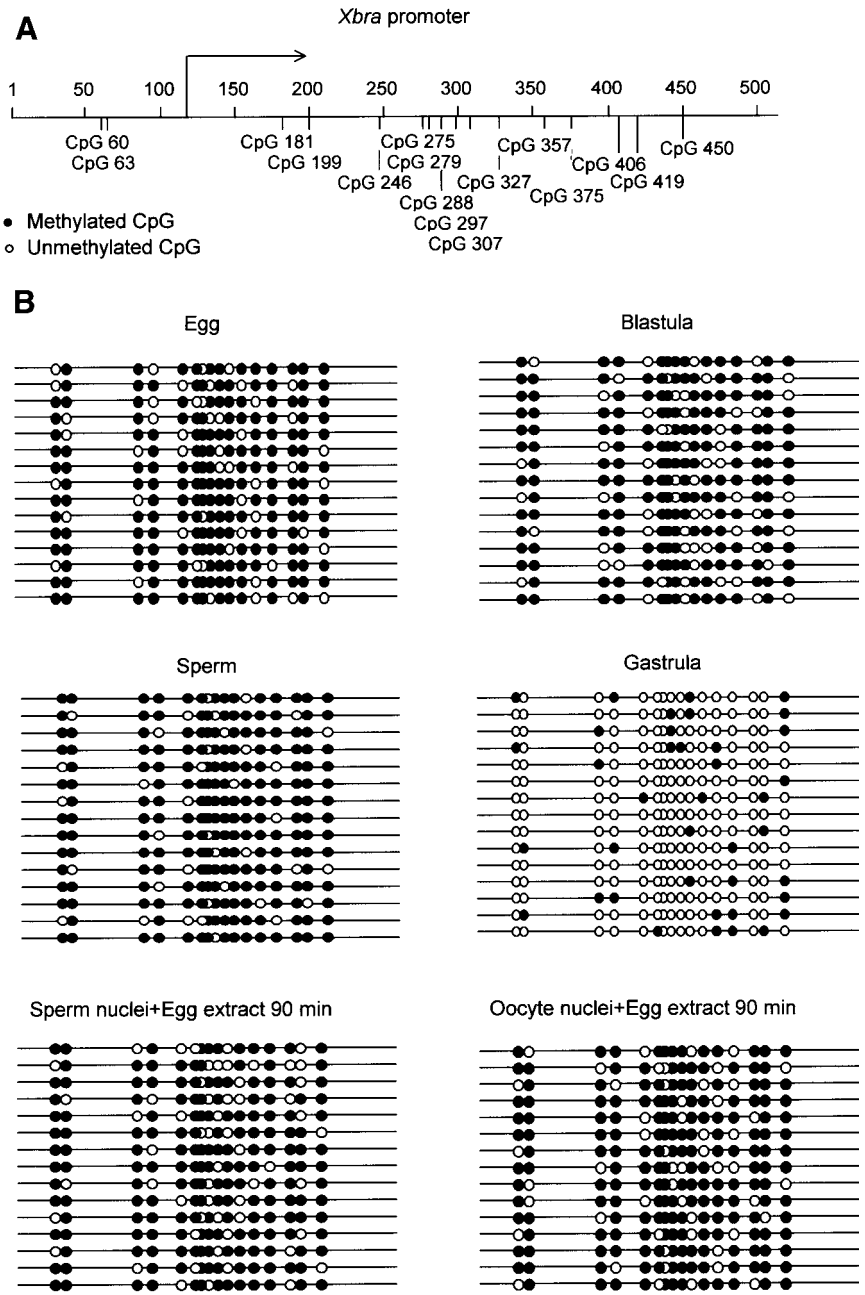

FIG. 3. The *Xbra* promoter becomes demethylated during gastrula stages. (A) The CpG map for the 500-bp region surrounding the *Xbra* promoter is shown with the transcription initiation site indicated by an arrow. (B) Bisulphite sequencing analysis of this region from oocyte, sperm, and DNA derived from blastula- and gastrula-stage *Xenopus* embryos detects substantial demethylation of *Xbra* promoter in gastrula-stage embryos. The same region from sperm and oocyte nuclei which have been exposed to egg cytoplasmic extract remains highly methylated. Methylated CpGs are indicated by a filled circle and nonmethylated CpGs are indicated by an open circle. In each case, the sequencing of 15 independent clones is shown.

methylated in pre-MBT embryos, and loss of rDNA methylation accompanies the onset of ribosomal gene activity in gastrula embryos (Bird *et al.*, 1981; I.S. and R.R.M., unpublished observations). These results suggest that specific chromatin remodeling (due to gene activation) at promoter sequences leads to exclusion of Dnmt1 from the DNA template immediately after replication at MBT or, alternatively, loss of methylation may be due to a competition

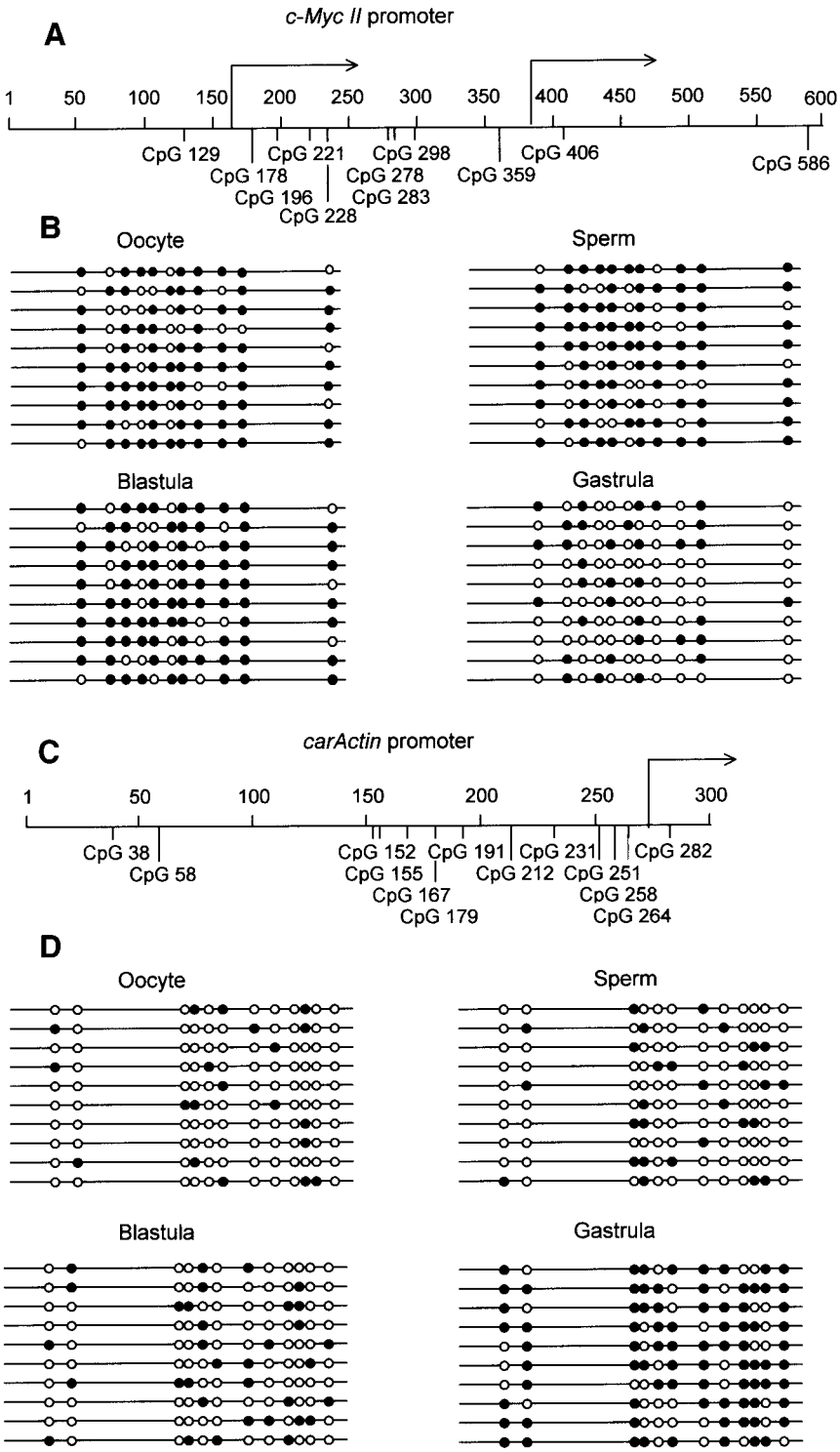

FIG. 4. The methylation pattern of the *c-Myc II* and *cardiac Actin* promoters are developmentally regulated. (A) The map of CpGs for the 600-bp sequence surrounding the *c-Myc II* promoter is shown with the transcription initiation sites illustrated by arrows. (B) Bisulphite sequencing analysis of this region during different stages of *Xenopus* development shows that *c-Myc II* promoter is undermethylated in gastrula-stage (12.5) embryos. In each case, 10 independent sequencing reactions are shown. Methylated CpGs are indicated by a filled circle, and nonmethylated CpGs are indicated by an open circle. (C) Map of CpGs for the 300-bp fragment surrounding *carActin* transcription start site (indicated by an arrow). (D) Sequencing of 10 independent clones from oocyte, sperm, blastula, and gastrula indicated that *cardiac actin* promoter in contrast to *Xbra* and *c-Myc II* is undermethylated in the early embryo but acquires methylated CpGs in stage 12.5 gastrula.

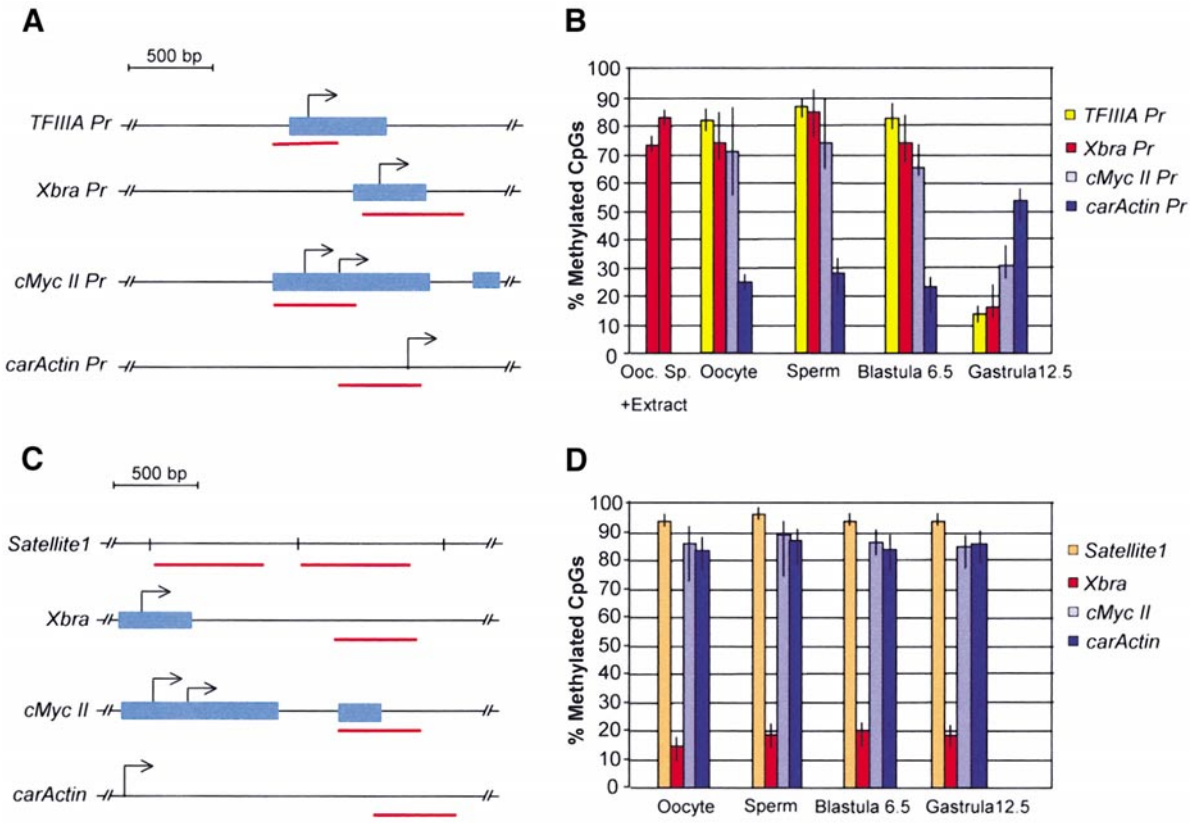

FIG. 5. Summary of bisulfite sequencing experiments. (A) Schematic representation of *TFIIIA*, *Xbra*, *c-Myc II*, and *cardiac Actin* promoters. The arrows indicate the transcription start site, blue boxes are CpG-island-like regions, the red lines show the fragments used for bisulfite sequencing. (B) Illustrated are the average values of 5mC content as detected by bisulfite sequencing and analysis of 10–15 individual clones on the 4 promoters in sperm, oocyte, blastula-, and gastrula-stage embryos. The error bars show the lowest and the highest Me-CpG values found in the individual clones. Note that TFIIIA, Xbra, and *c-Myc II* promoters undergo demethylation at gastrulation, while the *cardiac Actin* promoter becomes more methylated. (C) Schematic representation of Xbra, *c-Myc II*, and *cardiac Actin* coding regions and *Satellite I* repeats. The arrows indicate the transcription start sites, blue boxes are CpG island-like regions, the red lines show the fragments used for bisulfite sequencing. (D) Illustrated are the average values of 5mC content as detected by bisulfite sequencing and analysis of 10–15 individual clones on the 3 coding regions and *Satellite I* repeats in sperm, oocyte, blastula-, and gastrula-stage embryos. The error bars show the lowest and the highest Me-CpG values found in the individual clones. Note that there is no change in the 5mC levels of all four analyzed sequences.

between Dnmt1 and transcription factors for binding to promoter sequences (Matsuo *et al.*, 1998). It is possible that competition for promoter binding sites is shifted in favor of transcription factors around MBT since the levels of the Dnmt1 enzyme are particularly low at this stage of development (Stancheva and Meehan, 2000).

Overall, the relative changes in 5mC content that we observe during early *Xenopus* embryogenesis are much less dramatic than those seen in preimplantation mouse embryos, but at the same time, different to the stable methylation levels of repeat sequences and coding regions observed during zebrafish development (Martin *et al.*, 1999; Mayer *et al.*, 2000; Macleod *et al.*, 1999; Oswald *et al.*, 2000). Although DNA methylation may have a conserved role in transcriptional repression in vertebrates, it can be relatively plastic in its application to different developmental contexts. In general, the dynamics of methylation patterns in *Xenopus* can be related to the timing of zygotic activation of developmentally regulated genes. The transcription factors required for the expression of the early mesodermal marker gene *Xbra* and the housekeeping genes *c-Myc II* and *TFIIIA* are present and stored as maternal RNAs and proteins in the early embryo (Artinger *et al.*, 1997; Principaud and Spohr, 1991; Smith *et al.*, 1991; Taylor *et al.*, 1986). This allows rapid activation of *Xbra*, *c-Myc I*, *TFIIIA*, and other early genes at MBT by maternally activated signal transduction pathways when the decrease in the histone/DNA ratio and reduced genomic methylation levels favor transcription over repression.

Our results imply that premature gene activation in

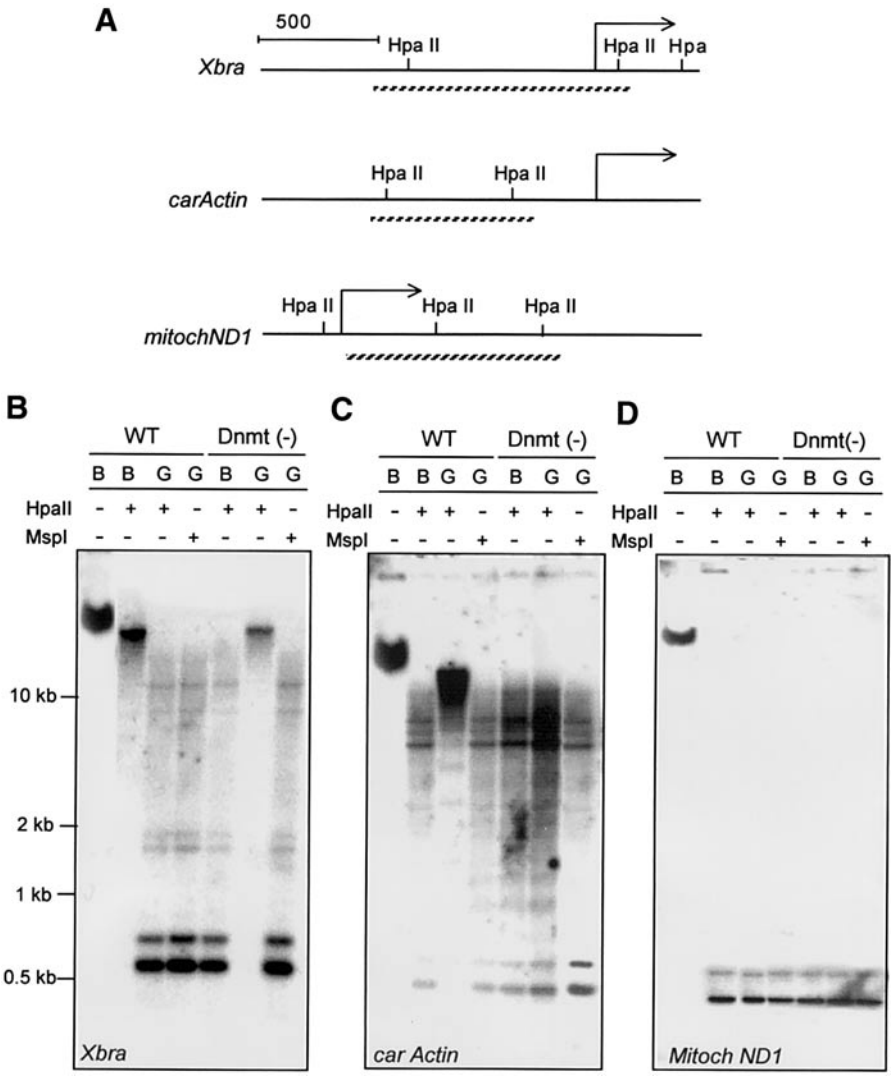

FIG. 6. Developmentally programmed changes in methylation of *Xbra* and *cardiac Actin* promoters are disrupted in maternal *xDnmt1*-depleted embryos. (A) Diagram indicating the *Hpa*II sites of the *Xbra*, *cardiac Actin*, and *mitochondrial ND1* regions that were analyzed by Southern blotting of genomic DNA from staged embryos. The broken line bars indicate the location of hybridization probes used in these experiments. DNA was isolated from staged wild-type (WT) and maternally *xDnmt1*-depleted [Dnmt1(−)] staged embryos and digested with either *Hpa*II restriction enzyme, methylation sensitive at CmCGG, or *Msp*I, methylation insensitive at the same sequence, as a control. (B) A Southern blot hybridized with a 1.5-kb probe derived from the *Xbra* promoter. Note that the promoter is completely hypomethylated in xDnmt1-depleted blastulae compared to the WT but undergoes abnormal *de novo* methylation in xDnmt1(−) gastrulae. (C) The same blot was rehybridized with a 2-kb *cardiac Actin* promoter probe. This promoter region fails to be methylated *de novo* in xDnmt1(−) gastrulae as compared to the WT, which suggests that the *de novo* methylation signal is disrupted in *xDnmt1*-depleted embryos. (D) The blot was also rehybridized with a mitochondrial DNA probe (*ND1*) as a control for complete restriction enzyme digestion. Mitochondrial DNA is never methylated and is equally digested by both *Hpa*II and *Msp*I.

xDnmt1-depleted embryos is specifically restricted to those genes that are normally activated at MBT in wild-type embryos (Stancheva and Meehan, 2000; Dunican, I.S., and R.R.M., unpublished observations). We did not observe ectopic activation of genes outside this development context. For example, the *cardiac Actin* promoter is maintained as an unmethylated sequence and is not expressed in the cleavage-stage embryos, perhaps because the transcription factors and upstream induction pathways needed for its activation do not appear until much later in development (Mohun *et al.*, 1986). Therefore, it is perhaps not surprising that ectopic expression of *cardiac Actin* is not observed in *xDnmt1*-depleted embryos (I.S. and R.R.M., unpublished observations). During gastrulation, some copies of this

promoter become methylated, which may reflect restricted expression of *cardiac Actin* gene in heart and somite muscle tissues (Fig. 5B). On a similar note, despite hypomethylation of the *Trx* family of retroposons in *xDnmt1*-depleted embryos, no ectopic *Trx* transcripts were observed (I.S. and R.R.M., unpublished observations). It is clear from our data that loss of methylation at CpG islands cannot account for the drop in DNA methylation (40%) that we observe at MBT. If we assume that there are 20,000 CpG islands in the *Xenopus* genome with an average size of 500 bp, then this would correspond to about 1% of all CpGs. This raises the question as to what other type of sequences or domains become hypomethylated at this stage of *Xenopus* development. There is a transition from random to specific anchorage of the nuclear matrix when chromatin domains become active at MBT (Vassetzky *et al.*, 2000). In addition, replication origin usage changes at the same time (Mechali, 2001). It is possible that epigenetic marks, via chromosomal remodeling mechanisms, play a role in the determination of active chromosomal territories in the embryo. Future experiments will address the nature of sequences that become hypomethylated at MBT.

The significance of preexisting early methylation patterns becomes apparent in *Xenopus* embryos transiently depleted from maternal xDnmt1. Loss of methylation during the cleavage stages of blastula embryos leads to irreversible changes in the methyl-CpG patterns of some gene promoters (*Xbra* and *cardiac Actin)* later in development. In effect, the *Xbra* promoter is aberrantly methylated and the *cardiac Actin* promoter is hypomethylated during gastrula stages. This suggests that the normal regulation of the *de novo* methyltransferase activities has been altered in Dnmt1-depleted embryos, which distorts their developmental program. It will be of interest to determine which signaling pathways are involved in the regulation of these activities during embryogenesis.

In zebrafish, which is similar to *Xenopus* with respect to the timing of zygotic gene activation, it is also not clear whether regulation of gene expression during development is accompanied by a remodeling of methylation patterns at specific loci. To date, candidate promoters regions have not been analyzed by bisulfite sequencing in zebrafish (Macleod *et al.*, 1999). However, embryos grown in the presence of demethylating drug 5-azacytidine display very similar developmental defects to those observed in *Xenopus* (Martin *et al.*, 1999; Stancheva and Meehan, 2000). It is possible that regulation of developmental gene activation by DNA methylation will be observed in additional nonmammalian animal species.

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